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(54) Title: NURR1 TRANSCRIPTION FACTOR I-BOX MUTANTS HAVING MONOMERIC TRANSCRIPTIONAL ACTIVA-
TION ACTIVITY

(57) Abstract: The invention relates to the finding that the Nurr1 transcription factor, which forms a heterodimer with the retinoid X receptor, can be mutated in the I box region such that dimerisation does not occur while Nurr1 transcriptional activation activity is retained. The invention provides Nurr1 peptides with such I box mutations, as well as assay methods for modulators which affect the monomeric activity of Nurr1.

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NURR1 TRANSCRIPTION FACTOR I-BOX MUTANTS HAVING MONOMERIC
TRANSCRIPTIONAL ACTIVATION ACTIVITY

Field of the invention.

5

The present invention relates to novel mutants of the Nurrl transcription factor, and to their use in assays to identify modulators of Nurrl activity.

10 Background of the invention.

Nurrl is a transcription factor belonging to the superfamily of nuclear receptors. This receptor family consists of the receptors for steroid hormones, retinoids, thyroid hormone,
15 vitamin D, and a large group of receptors whose ligands are unknown. The receptors with unidentified ligands are referred to as orphan nuclear receptors (Truss and Beato 1993, Mangelsdorf et al. 1995).

20 Most nuclear receptors act as dimers, either as homodimers or as heterodimers. Two dimerization interfaces have been identified in the DNA-binding domain and the ligand-binding domain (LBD), respectively. The dimerization interface in the LBD - the I-box - has been mapped to a region in the carboxyl-
25 terminal part of the LBD, corresponding to helix 10 in the canonical nuclear receptor LBD structure (Perlmann et al. 1996, Lee et al. 1998). This region is well conserved among several dimerizing receptors, including Nurrl (Fig. 1) and Nor
1. In Nurrl, the I-box is the region at amino acids 524-556
30 of the full length protein.

Nurrl is able to bind to DNA as a monomer (Wilson et al. 1991). In addition, Nurrl forms heterodimers with retinoid X receptor (RXR) and, in contrast to most other RXR-

heterodimers, this dimer can be activated by retinoids (Perlmann and Jansson 1995, Forman et al 1995, Zetterström et al. 1996).

5 The functional roles of the Nurrl monomer and Nurrl-RXR heterodimer *in vivo* remain to be elucidated. Nevertheless, expression studies relating to Nurrl have shown that Nurrl, as the closely related receptors NGFI-B and Norl, is predominantly expressed in the CNS (Milbrandt 1988, Law et al. 1992, Ohkura et al. 1994, Zetterström et al. 1996). In the
10 CNS, it is detected e.g. in the cortex, hippocampus, hypothalamus, and in the dopaminergic neurons of the substantia nigra and ventral tegmental area (Zetterström et al. 1996).

15 Nurrl is expressed in the developing and adult midbrain dopaminergic neurons. Studies have shown that Nurrl plays a critical role in the development of these neurons as mice whose Nurrl gene has been inactivated (Nurrl -/- mice) fail to generate the midbrain dopamine cells (Zetterström et al. 1997, Saucedo-Cardenas et al. 1998, Castillo et al. 1998, Wallén et al. 1999). These neurons degenerate in patients with
20 Parkinson's disease, and so Nurrl may be important in the development of this disease. Nurrl seems also to play an important role in other disorders of dopamine transmission as mutations in the coding sequence of Nurrl have been identified in schizophrenic and manic-depressive patients (Buervenich et al. 2000).

30 Therefore, due to its potential role in dopaminergic neurons, Nurrl is a potential target for pharmacological treatment of disorders involving dopamine transmission. Agents, for example small lipophilic compounds, which interact with Nurrl

and affect its transcriptional activation function or DNA binding properties have potential therapeutic utility.

Screens for compounds which modulate nuclear receptors are often based upon reporter gene activation assays performed in cultured cells. In such assays, it would be difficult to distinguish between agents which affect the activity of the Nurrl-RXR heterodimer, and those which are specific to the Nurrl monomer. Thus, using wild-type Nurrl in such an assay would require further testing of agents to determine which complex is modulated. Because it is possible that Nurrl and Nurrl-RXR complexes have distinct roles *in vivo*, it would be desirable to be able to distinguish more efficiently between the monomer and the heterodimer complex.

The ligand binding domain of Nurrl, encompassing the I-box, is structurally complex. It has been shown that truncating a region including the I-box of Norl interferes with the monomeric activity of this receptor (Labelle et al, Oncogene 18(21):3303-8, 1999). By analogy, it might be expected that similar disruptions to Nurrl would likewise disrupt the function of this protein.

Disclosure of the invention.

The present inventors have surprisingly discovered that Nurrl polypeptides with particular mutations in the I-box region are unable to dimerise with RXR but surprisingly retain the ability to promote gene expression as monomers. This is particularly notable since the ligand binding domain of Nurrl includes transcriptional activation functions, so that the removal of the ability to dimerise could lead to removal of transcription activation function.

The present invention thus provides a Nurrl polypeptide which in comparison to a wild-type Nurrl polypeptide has at least one amino acid difference, which is unable to promote RXR-mediated transactivation but which retains the ability to promote gene expression as a monomer, said difference being in the I-box region.

Preferred differences occur at one or more (e.g. two or three or four) I-box residues at residues 524 to 566, for example in the region of 553 to 563. Preferred residues are those selected from the positions 554, 555, 556, 557, 558, 559, 560, 561 and 562 of Nurrl.

Although not all the single substitutions in this region had the desired effect, i.e. still formed a heterodimer, the data show that all the polypeptides retained the ability to promote gene expression as a monomer, and establish the principle that a number of different types of changes can be made to the I-box region and retain this differential activity.

The differences may be any substitution, insertion or deletion which provides for the loss of RXR heterodimerization.

Substitutions include substitution of any amino acid (apart from alanine) by alanine, substitutions which result in a change from a positive to a negative charge (e.g. E561K), or vice versa, or changing an uncharged amino acid into a charged or more polar amino acid (e.g. L562K). The alteration of amino acids in a protein by routine protein engineering techniques is well known in the art, and having demonstrated the various changes shown in the accompanying examples, a person of skill in the art will be able to make and test further similar changes without undue burden. Deletions may be of for example from 1 to 10, such as 1, 2, 3, 4 or 5 residues of the I-box region. The deleted residues may be

adjacent or located between other I-box residues which are retained. Insertions may be of for example from 1 to 10, such as 1, 2, 3, 4 or 5 residues, of any amino acid, into the I-box region. Where more than 1 residue is inserted, the residues
5 may be contiguous or located between different existing I-box residues. Any one of the 20 naturally occurring amino acids may be inserted. Preferably the inserted residues will be non-aliphatic, e.g. alanine, leucine, valine, isoleucine, glycine, serine, lysine or the like.

10 Combinations of substitutions, insertions and deletions may be made, e.g. one or more substitutions and one or more deletions. Where a combination of two of these categories of changes is made, the number of amino acid changes may be for
15 example up to a total number of changes from 2 to 10, such as 2, 3, 4 or 5.

Examples of polypeptides of the invention include those in which at least two, preferably at least three adjacent
20 residues are altered, preferably substituted. Such substitutions may be different or the same, e.g. all to alanine.

Specific examples of such polypeptides include the
25 polypeptides Nurrl KLL(554-556)AAA, GKL(557-559)AAA, PEL(560-562)AAA or P560A.

Substitutions at these positions are demonstrated to abolish the ability of the Nurrl polypeptide to promote RXR-mediated
30 transactivation. However, the ability of the polypeptides to activate reporter gene expression as monomers is retained.

The invention further provides a vector comprising a nucleic acid sequence encoding a Nurrl polypeptide of the invention.

The invention further comprises an assay method for determining if an agent is a modulator of Nurrl activity, said method comprising:

providing a Nurrl polypeptide of the invention together
5 with a putative modulatory agent; and

determining whether or not said agent is able to modulate the transcriptional activity of said polypeptide.

Agents which modulate transcriptional activity may do this by
10 binding to the polypeptide in a manner which allows the polypeptide to retain DNA binding activity and which results in a loss or increase of transcriptional activation, or the agent may be one that modulates DNA binding activity itself.

15 Generally, assays of the invention fall under the classes of reporter gene assays, coactivator interaction assays (two-hybrid assays) and DNA binding assays. By modulating the DNA binding activity of Nurrl polypeptides of the invention, it may be inferred that the transcriptional activity of the
20 protein is affected.

Nurrl is important in developing dopamine cells and may also have functions in adult dopamine neurons. Thus modulators found by the assay of the invention may have use in the
25 treatment of Parkinson's disease, schizophrenia, drug addiction, attention deficit hyperactivity disorder (ADHD), manic depression, and other conditions related to aberrant activity of dopamine neurons.

30 Brief Description Of The Drawings.

Figure 1 shows the structural and functional domains of nuclear receptors including the region important for dimerisation (the I-box). NT, amino-terminal domain; DBD, DNA-

binding domain; LBD, ligand-binding domain. The 11 amino acids (SEQ ID NO:1) of the Nurrl I-box altered in the accompanying examples are shown underlined.

5 Figure 2 shows the effect of Nurrl I-box mutations on the ability to promote RXR-mediated transactivation. The wild-type and mutated Nurrl derivatives were transfected in JEG3 cells. The ability to transactivate was assessed using a luciferase reporter gene driven by three copies of the hRAR β 2 promoter
10 RARE (β RE) upstream of a thymidine kinase promoter.

Figure 3 shows that the I-box mutations have no effect on the monomeric activity of Nurrl. The monomeric activity was examined in 293 cells. The full-length receptor derivatives
15 were studied using a reporter gene regulated by three copies of the NGFI-B binding site (NBRE; fig. A).

The activities of the wild-type and mutated ligand-binding domains fused to Gal4 DNA-binding domain were examined on a
20 reporter containing four Gal4-binding sites upstream of a minimal thymidine kinase promoter (fig. B). Essentially identical results were obtained when the experiments were carried out in JEG3 cells.

25 Figure 4 shows the influence of Nurrl I-box mutations on interaction with RXR in JEG3 cells. The wild-type and mutated Nurrl ligand-binding domains were fused to the Gal4 DNA-binding domain and the interaction with RXR fused to the VP16 activation domain was examined as described by Perlmann and
30 Jansson 1995.

Figure 5 shows the effect of I-box mutations on Nurrl-RXR heterodimerization on DNA. The ability of the Nurrl

derivatives to bind DNA as a heterodimer with RXR was assessed *in vitro* using the gel-shift assay as described by Castro et al. 1999.

- 5 Radioactively labelled hRAR β 2 promoter RARE (β RE) was used as the probe.

Detailed Description Of The Invention.

- 10 In the present invention, the Nurrl polypeptide preferably is the murine polypeptide. The amino acid sequence of the murine polypeptide, which is 598 amino acids in size, is available as Genbank accession number S53744. Other Nurrl polypeptides are known, and may also be used. For example, the human and rat
- 15 sequences are highly conserved to the murine sequence and have identical numbering of residues in the I-box region. The human Nurrl, also called NOT, is available as Genbank accession number NM006186. Rat Nurrl (RNR) is available as Genbank accession number U72345. All of these have been cloned and
- 20 are thus available in the art. Due to the very high level of identity of these polypeptides, they may equally be used in the present invention, and reference herein to Nurrl includes such other species forms of the polypeptide.
- 25 Our data herein indicate that the transcriptional activation activity of the I-box altered Nurrl is retained by fragments of Nurrl which have a deletion of their N-terminal region, which also has some transcriptional activating activity. Thus, reference herein to a Nurrl polypeptide also includes
- 30 fragments which retain the ability to activate transcription through the ligand binding domain region. Such fragments may comprise at least about 200, preferably at least about 250 amino acids of the full length Nurrl sequence. This is

demonstrated in Figure 3B herein, where a Nurrl polypeptide of 246 amino acids is fused to the Gal4 DNA binding domain, and it is shown that this fusion is able to activate transcription from a reporter containing Gal4 binding sites upstream of a tk promoter.

Where Nurrl polypeptides whose sequences are not on public databases are required, e.g. from other species, these may be obtained by routine cloning methodology. For example, a library of cDNA from a mammalian or other species may be made and probed with all or a portion of a DNA sequence encoding Nurrl under conditions of medium to high stringency.

For example, hybridizations may be performed, according to standard methods well known as such in the art using a hybridization solution comprising: 5X SSC (wherein SSC is 0.15 M sodium chloride; 0.15 M sodium citrate; pH 7), 5X Denhardt's reagent, 0.5-1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization, filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes - 1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30 minutes.

Clones identified as positive may be examined to identify open reading frames encoding Nurrl. It may be necessary to combine more than one clone to achieve a full length open reading frame, as would be understood by the person skilled in art. Clones may then be expressed in a heterologous expression system, e.g. in bacteria or yeast and the protein purified by techniques known in the art.

PCR cloning methods may also be used, based on PCR primers selected for sequences which are conserved between currently known Nurrl genes found in rat, mouse, human or other species.

5 In further aspects the invention provides a nucleic acid encoding a Nurrl polypeptide of the invention, and a vector comprising such nucleic acid. The vector is preferably an expression vector, wherein said nucleic acid is operably
10 linked to a promoter compatible with a host cell. The invention thus also provides a host cell which contains an expression vector of the invention. The host cell may be bacterial (e.g. E.coli), insect, yeast or mammalian (e.g. hamster or human). The vector may be any suitable DNA or RNA
15 vector.

Host cells of the invention may be used in a method of making a Nurrl polypeptide of the invention as defined above which comprises culturing the host cell under conditions in which
20 said polypeptide or fragment thereof is expressed, and recovering the polypeptide in isolated form. The polypeptide may be expressed as a fusion protein.

Examples of suitable vector systems include bacterial vectors
25 such as pBR322, pUC18 and pUC19, yeast expression vectors, and mammalian vectors, for example vectors based on the Moloney murine leukaemia virus (Ram, Z et al., Cancer Research (1993) 53; 83-88; Dalton and Triesman, Cell (1992) 68; 597-612. These vectors contain the murine leukaemia virus (MLV) enhancer
30 cloned upstream at a β -globin minimal promoter. The β -globin 5' untranslated region up to the initiation ATG is supplied to direct efficient translation of the cloned protein.

"Operably linked" means joined as part of the same nucleic

acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter.

Polypeptides and nucleic acids of the invention may be in
5 isolated form, free or substantially free of material with
which they are naturally associated such as other polypeptides
or nucleic acids with which they are found in the cell. The
polypeptides and nucleic acids may of course be formulated
with diluents or adjuvants and still for practical purposes be
10 isolated - for example the polypeptides will normally be mixed
with gelatin or other carriers if used to coat microtitre
plates for use in immunoassays. Polypeptides may be
glycosylated, either naturally or by systems of heterologous
eukaryotic cells, or they may be (for example if produced by
15 expression in a prokaryotic cell) unglycosylated.
Polypeptides may be phosphorylated and/or acetylated.

A polypeptide of the invention may also be in a substantially
purified form, in which case it will generally comprise the
20 polypeptide in a preparation in which more than 90%, e.g. 95%,
98% or 99% of the polypeptide in the preparation is a
polypeptide of the invention.

Assay methods practiced according to the invention may be
25 arranged in any suitable format known as such to those of
skill in the art. For example, an assay may be configured to
measure the transcriptional-activating properties of a
polypeptide of the invention, by providing a reporter gene
construct comprising a promoter region containing one or more
30 (e.g. two, three, four or five) elements to which Nurrl
polypeptides may bind and in doing so, activate transcription
of the reporter gene. Examples of suitable constructs are
described in the accompanying examples.

For determination of transcriptional activation by a Nurrl-RXR dimer, the hRAR β 2 promoter RARE may be used. In contrast, the NGFI-B response element (NBRE) described herein below may be used as a target for monomers of Nurrl polypeptides to bind to.

These elements may be linked to a suitable transcription initiation region, such as the thymidine kinase gene initiation region, such that transcription is activated by binding of Nurrl to its cognate binding region(s).

Many suitable reporter genes are known as such in the art, for example luciferase, green fluorescent protein, chloramphenicol acetyl transferase, beta galactosidase, and the like.

Assays of the invention based on the binding of a Nurrl polypeptide to its cognate DNA binding sequence may be conducted *in vitro*, and may take any suitable form known to those of skill in the art.

Generally, one or both of the DNA and the polypeptide will carry a detectable label, such as a fluorescent label. The polypeptide and DNA will be brought into contact with each other under conditions suitable for binding to occur, and then the amount of binding in the presence of a putative modulator determined and compared to a suitable control, e.g. the amount of binding in the absence of modulator or a pre-selected modulator with desirable properties.

The binding of DNA to polypeptide can be measured in a number of ways known as such to the skilled person. For example, one or other component may be immobilized on a solid support, and the other brought into contact with it, incubated and then unbound material rinsed away prior to measurement.

One example of an assay format is dissociation enhanced lanthanide fluorescent immunoassay (DELFI A). This is a solid phase based system for measuring the interaction of two macromolecules. Typically one molecule is immobilized to the surface of a multi well plate and the other molecule is added in solution to this. Detection of the bound partner is achieved by using a label consisting of a chelate of a rare earth metal. This label can be directly attached to the interacting molecule or may be introduced to the complex via an antibody to the molecule or to the molecules epitope tag. Alternatively, the molecule may be attached to biotin and a streptavidin-rare earth metal chelate used as the label. The rare earth metal used in the label may be europium, samarium, terbium or dysprosium. After washing to remove unbound label, a detergent containing low pH buffer is added to dissociate the rare earth metal from the chelate. The highly fluorescent metal ions are then quantitated by time resolved fluorimetry. A number of labeled reagents are commercially available for this technique, including streptavidin, antibodies against glutathione-S-transferase and against hexahistidine.

Compounds which target nuclear receptors are of substantial commercial significance in the pharmaceutical industry. Generally, nuclear receptors can be targeted by compounds which are small lipophilic molecules such as steroids, thyroid hormone, retinoids, prostanoids, fatty acids, fatty acid derivatives and numerous small synthetic hydrophobic compounds. Thus potential modulator compounds which may be used in assays of the invention may be natural or synthetic chemical compounds used in drug screening programmes of these classes.

Candidate modulator compounds, including both agonists and antagonists, obtained according to the method of the invention

may be prepared as a pharmaceutical preparation. Such preparations will comprise the compound together with suitable carriers, diluents and excipients. Such formulations form a further aspect of the present invention. The formulations may be used in methods of treatment of various conditions associated with aberrant function of dopamine neurons, such as those mentioned herein above.

The amount of putative inhibitor compound which may be added to an assay of the invention will normally be determined by trial and error depending upon the type of compound used. Typically, from about 0.01 to 100 μ M concentrations of putative inhibitor compound may be used, for example from 0.1 to 10 nM.

The invention will now be described in detail with reference to the following examples.

Examples.

MATERIALS AND METHODS

The Nurrl I-box mutants were generated using the GeneEditorTM *in vitro* Site-Directed Mutagenesis System (Promega) according to the manufacturer's instructions. In short, pCMX-Nurrl expression vector encoding the full-length mouse Nurrl was used as the template. Oligonucleotides with 21-40 nucleotides containing the desired mutation were hybridised to the denatured template, extended with T4 DNA polymerase and ligated with T4 DNA ligase. The oligonucleotides used were:

for KLL(554-556)AAA:

5'-CCCAACTACCTGTCTGCAGCGGCGGGGAAGCTGCCAGAAC-3' (SEQ ID NO:2);

for GKL(557-559)AAA:

5'-CTGTCTAAACTGTTGGCGGCGGCCAGAACTCCGCACC-3' (SEQ ID NO:3);

for PEL(560-562)AAA:

5 5'-CTGTTGGGGAAGCTGGCAGCAGCCCCGCACCCTTTGCAC-3' (SEQ ID NO:4);

K558A:

5'-AAACTGTTGGGGGCGCTGCCAGAACTC-3' (SEQ ID NO:5);

10 P560A:

5'-TTGGGGAAGCTGGCAGAACTCCGCACC-3' (SEQ ID NO:6);

E561A:

5'-AAGCTGCCAGCACTCCGCACC-3' (SEQ ID NO:7); and

15

L562A:

5'-AAGCTGCCAGAAGCCCGACCCTTTGC-3' (SEQ ID NO:8).

20 The bases coding for the alanines are underlined. The bacterial colonies obtained after transformation were screened by direct sequencing.

25 The influence of the I-box mutations on Nurrl transcriptional activity and on the ability to interact with RXR was examined as described by Perlmann and Jansson 1995, Zetterström et al. 1996, and Castro et al. 1999. Nurrl-RXR heterodimer-mediated transactivation was studied in human chorion carcinoma JEG3 cells. The cells were maintained in minimum essential medium supplemented with 10% fetal calf serum. Transfections were
30 performed in quadruplicates in 24-well plates using the calcium phosphate precipitation method. The cells were plated 24 h prior to the transfection. Each well was transfected with 100ng of the expression vector for the Nurrl variants, 100ng of a reporter plasmid, and 200ng of β galactoside plasmid

that was used as an internal control for transfection efficiency. The luciferase reporter used was driven by three copies of the retinoid acid response element (RARE) of the human retinoid acid receptor β 2 (hRAR β 2) gene promoter (β RE) upstream of the thymidine kinase promoter. 6-8 hours after transfection, the cells received fresh medium supplemented with 10% charcoal-stripped fetal calf serum and RXR ligand (SR11237;1 μ M). The cells were harvested 24 hours later and lysed. The cell extracts were assayed for luciferase and β galactosidase activity. The ability of the Nurrl mutants to interact with RXR was examined using the mammalian two-hybrid assay. The ligand-binding domains (amino acids 353-598; LBD) of both wild-type and mutated Nurrl variants were cloned in frame with the yeast Gal4 DNA-binding domain (amino acids 1-147) in the pCMX-Gal4 expression vector (Perlmann and Jansson, 1995). JEG3 cells were cotransfected with pCMX-Gal4-Nurrl-LBD derivatives and pCMX-VP16-RXR containing the herpes simplex VP16 trans-activation domain followed by the complete coding sequence of human RXR α . A reporter gene with four copies of the Gal4-binding sites was used.

The ability of the mutants to activate reporter gene expression as monomers was studied in human embryonic kidney 293 cells. The cells were maintained in Dulbecco's modified Eagles medium with 10% fetal calf serum. Transfections were carried out as with JEG3 cells except that a reporter regulated by three copies of the NGFI-B (nerve growth factor inducible-B) response element (Wilson et al., 1991) (NBREs) was used. The transcriptional activities of the mutated ligand-binding domains were assessed as fusions to the Gal4 DNA-binding domain and a reporter gene with four copies of the Gal4-binding sites was used. All the transfection experiments were performed in quadruplicate dishes and each experiment has been repeated at least twice with essentially identical

results. The results of a representative experiment are shown.

The DNA-binding experiments were carried out as described by Castro et al. 1999. Briefly, Nurrl and RXR proteins for gel mobility shift assays were produced by coupled *in vitro* transcription and translation in reticulocyte lysates according to the manufacturer's instructions (TNT Quick Coupled Transcription/Translation SystemTM; Promega). The proteins were incubated in a binding buffer containing 10mM Tris (pH 8.0), 40mM KCl, 0.05% NP-40, 6% glycerol, 1mM DTT, 0.2mg poly(dI-dC) and protease inhibitors. β RE-probe (agcttaaggGGTTCACCGAAAGTTCActcgcat; SEQ ID NO:9) was labelled with ³²P by fill-in reaction using Klenow fragment. After addition of the probe, the reactions were incubated on ice for 20 min. Protein-DNA complexes were resolved by electrophoresis on 4% non-denaturing polyacrylamide gel in 0.5 x TBE. After electrophoresis, the gels were dried for autoradiography.

RESULTS

Several of the Nurrl I-box amino acids were mutated either in combination or individually and the effects of these mutations were studied on RXR dimerization. First, three amino acid alanine substitutions were introduced to the I-box [KLL(554-556)AAA, GKL(557-559)AAA, and PEL(560-562)AAA]. All these mutations abolished the ability of Nurrl to promote RXR-mediated transactivation (Fig. 2). The ability to activate reporter gene expression as monomers was, however, intact (Fig. 3). In addition to the triple mutants, we created four individual alanine substitutions (K558A, P560A, E561A, and L562A) in order to examine the contributions of these residues on Nurrl-RXR dimerization. Mutation of the Pro560 to alanine

abolished the ability of Nurrl to activate reporter gene expression as a dimer with RXR but had no effect on monomeric activity (Fig 2 and 3A). Conversion of Lys558 to alanine had only a modest effect on Nurrl/RXR heterodimer-mediated transactivation. The ability of Nurrl to activate the Nurrl-RXR heterodimer-regulated reporter gene was reduced by the substitution of Leu562 by alanine but to a lesser extent than by the P560A mutation. Conversion of Glu561 to alanine had no effect. Thus, the three-residue alanine substitutions and the P560A mutation abolished the ability of Nurrl to promote RXR-mediated transcription.

N-terminal truncations of the full length protein also retained transcription activation activity (Fig 3B).

The mutations created could abolish Nurrl-RXR heterodimer-mediated reporter gene expression either by preventing Nurrl from dimerizing with RXR or by switching Nurrl into a non-permissive RXR partner. The effects of these mutations on heterodimerization were examined using the mammalian two-hybrid assay. All the mutations that blocked Nurrl-RXR-mediated reporter gene expression also prevented heterodimerization in cells (Fig. 4). The inability of these mutants to heterodimerize on DNA was confirmed *in vitro* by a gel-shift experiment (Fig 5). It was also observed that none of the mutations influenced monomeric DNA binding.

In conclusion, four different Nurrl mutants unable to heterodimerize with RXR were generated. Three of these involved three residue substitutions and one of them was a single amino acid mutation. All these mutants were able to bind DNA and to activate transcription as monomers. Therefore it is unlikely that these substitutions would have had any major effect on the overall structure of the Nurrl LBD or on

the ability to bind a putative ligand. Thus such mutants [e.g Nurrl KLL(554-556)AAA, GKL(557-559)AAA, PEL(560-562)AAA, and P560A] could serve as useful tools when searching for potential Nurrl activating ligands. The use of these mutants will allow to differentiate between solely Nurrl and Nurrl-RXR-heterodimer activating compounds.

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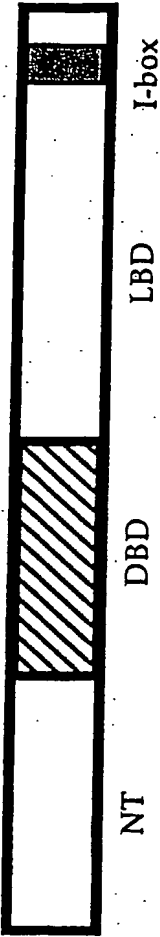
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CLAIMS:

1. A Nurrl polypeptide which in comparison to a wild-type Nurrl polypeptide has at least one amino acid difference,
5 which is unable to promote RXR-mediated transactivation but which retains the ability to promote gene expression as a monomer, said difference being in the I-box region.
2. A Nurrl polypeptide according to claim 1 wherein the
10 difference occurs at one or more I-box residues at residues 524 to 566.
3. A Nurrl polypeptide according to claim 2 wherein the difference occurs at a residue selected from the positions
15 554, 555, 556, 557, 558, 559, 560, 561 and 562 of Nurrl.
4. A Nurrl polypeptide selected from the group Nurrl KLL(554-556)AAA, GKL(557-559)AAA, PEL(560-562)AAA and P560A.
- 20 5. A Nurrl polypeptide according to any one of the preceding claims wherein the wild-type is murine Nurrl (Genbank accession number S53744).
6. A nucleic acid sequence encoding a Nurrl polypeptide of
25 the any one of the preceding claims.
7. An assay method for determining if an agent is a modulator of Nurrl activity, said method comprising:
providing a Nurrl polypeptide of any one of claims 1 to 5
30 together with a putative modulatory agent; and
determining whether or not said agent is able to modulate the transcriptional activity of said polypeptide.
8. An assay according to claim 6 which is a transcriptional

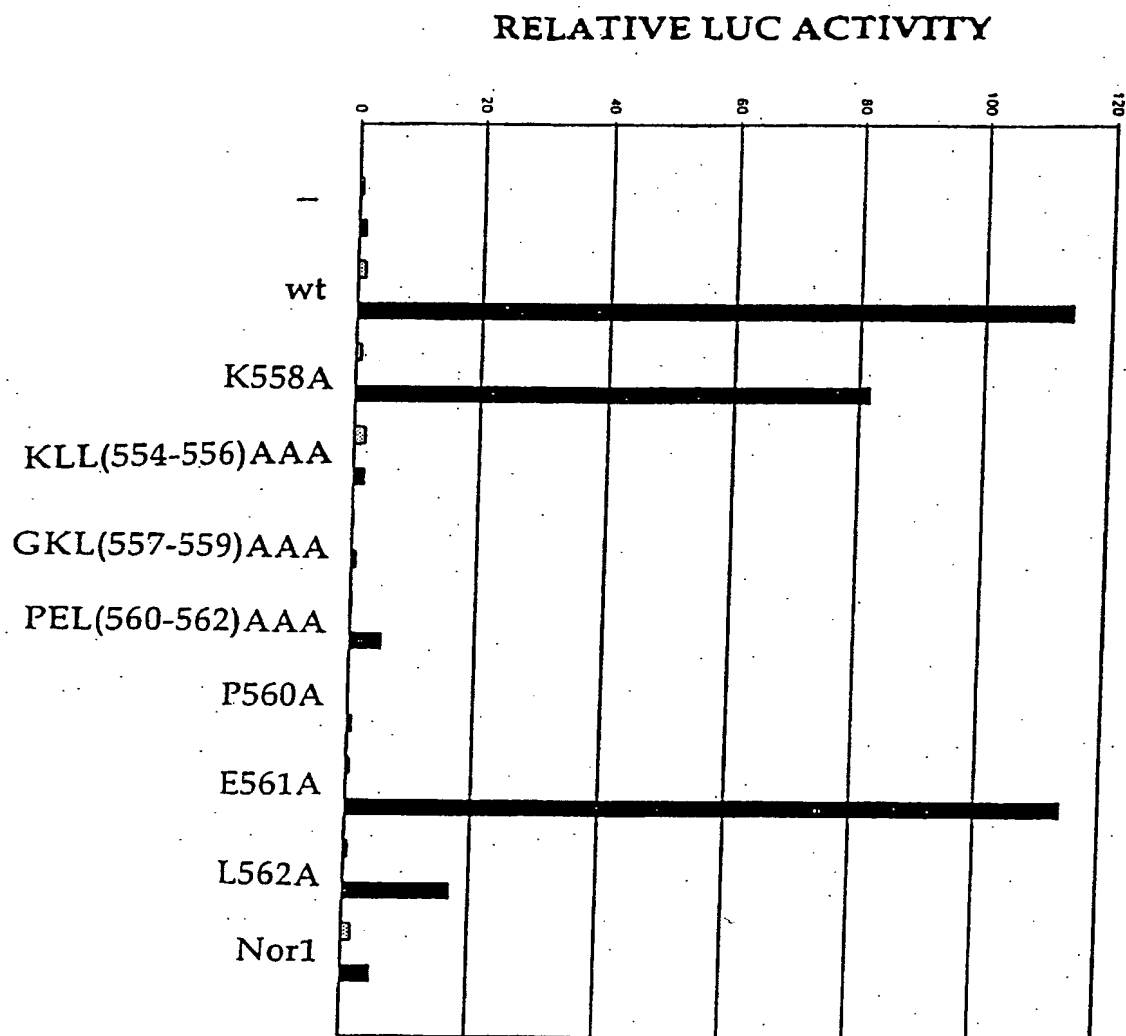
activation assay which uses a reporter gene construct.

FIGURE 1



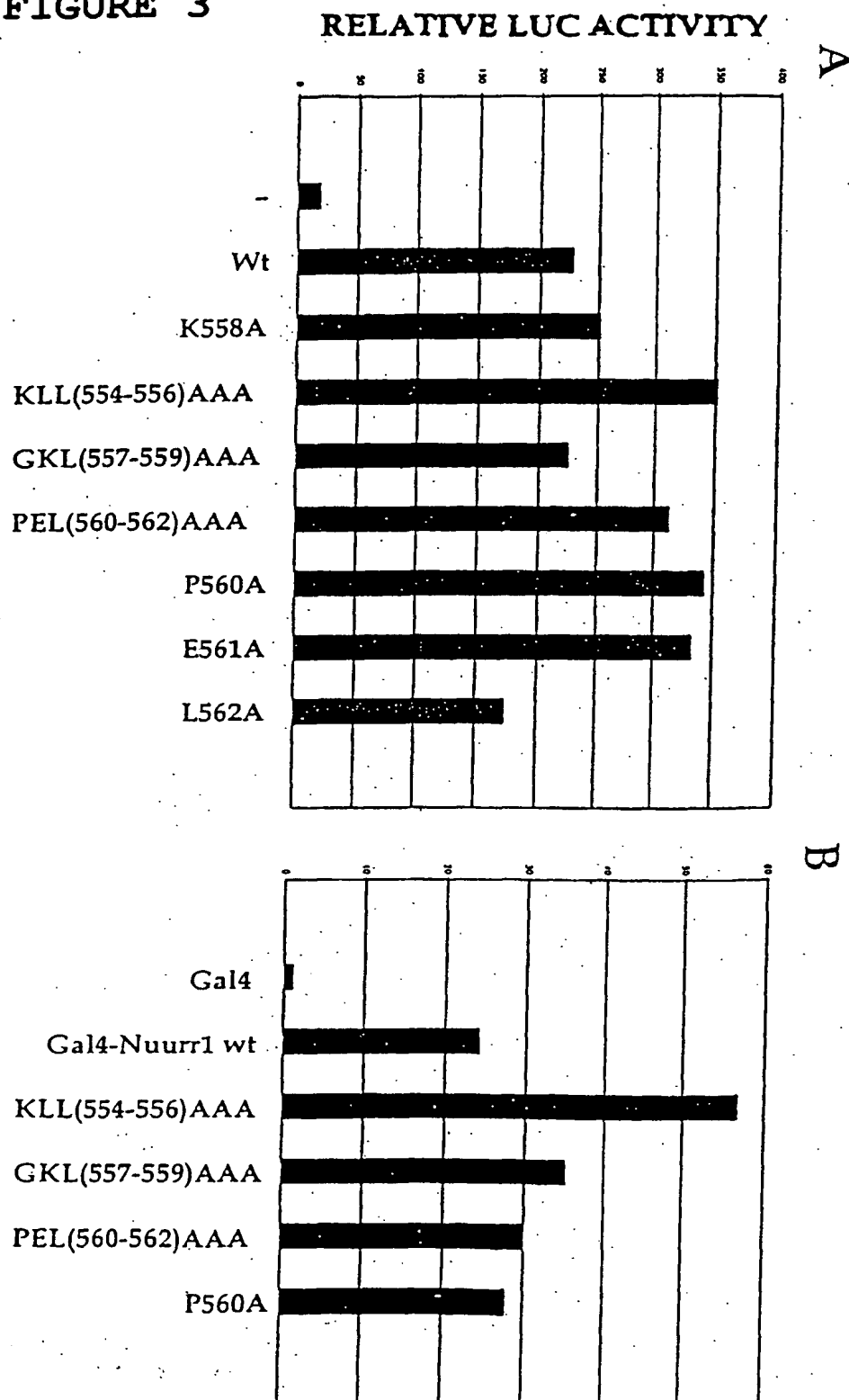
⁵⁵³ Nurr1 SKLLGKLP⁵⁶³ELR

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FIGURE 2**Best Available Copy**

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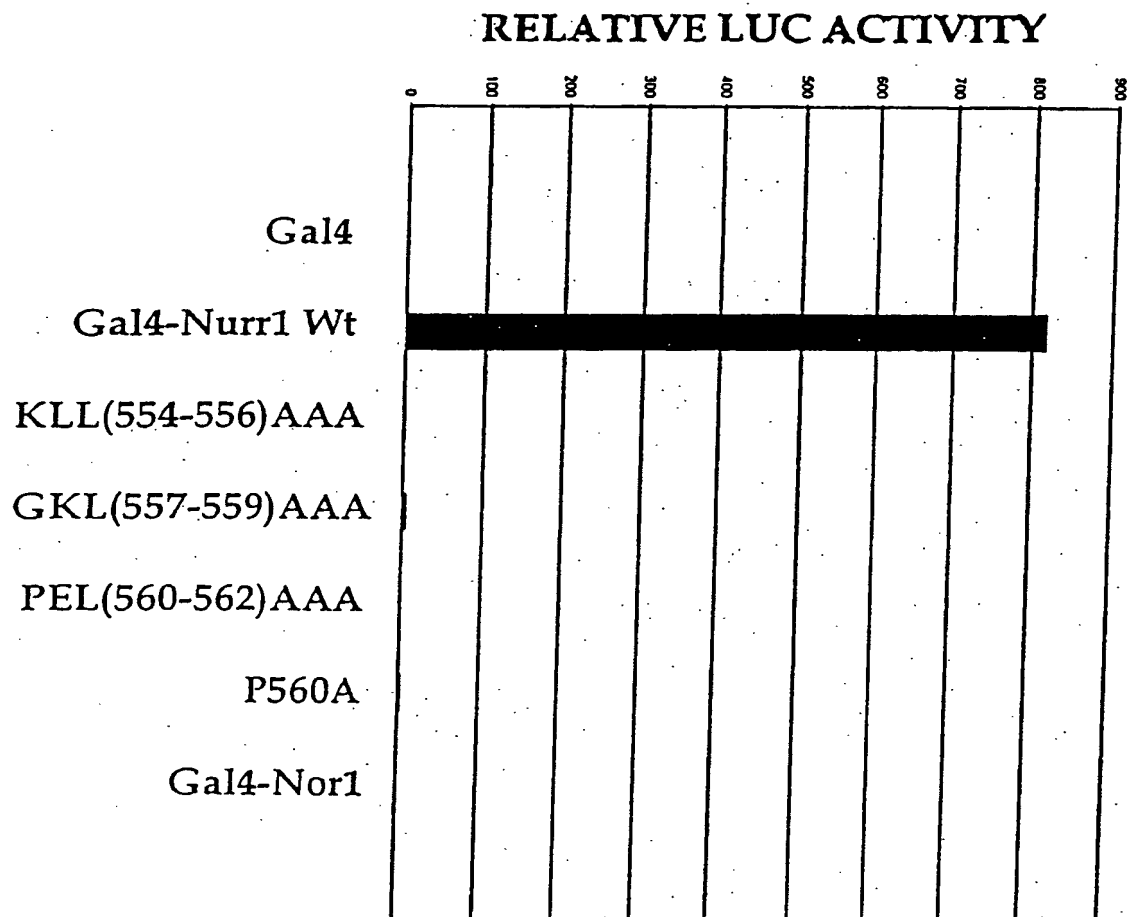
FIGURE 3



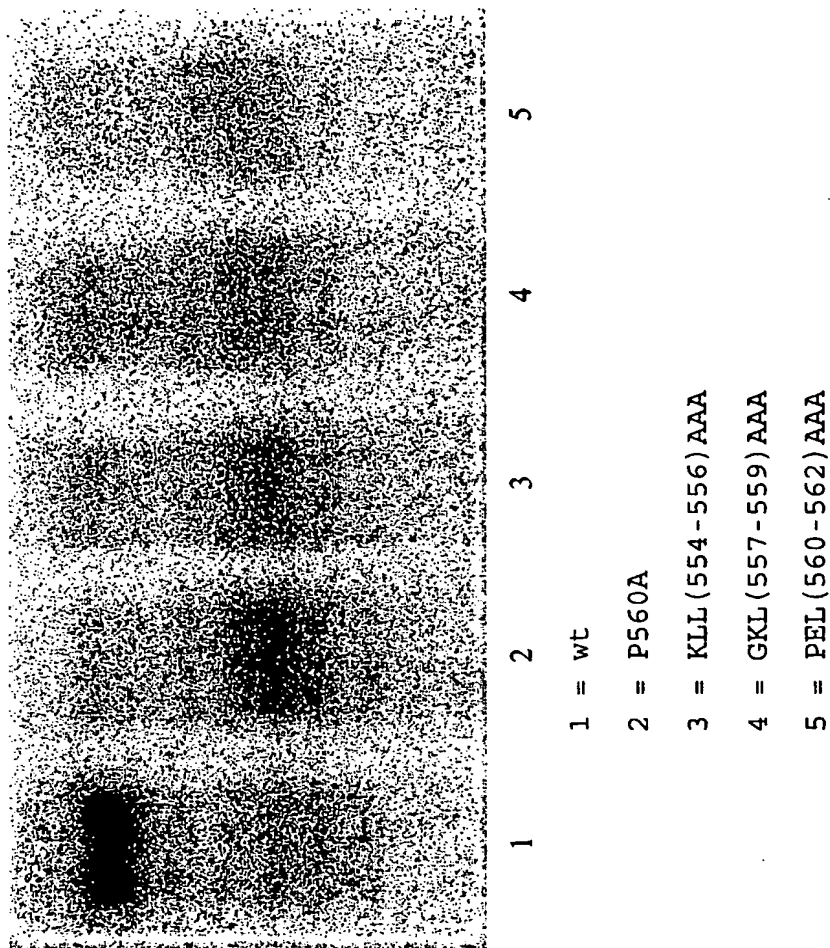
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FIGURE 4



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FIGURE 5

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INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/00744

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 C12N15/63 C07K14/705 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, SEQUENCE SEARCH, WPI Data, PAJ, BIOSIS, MEDLINE, CHEM ABS Data, EMBASE, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ZETTERSTROEM R H ET AL: "RETINOID X RECEPTOR HETERODIMERIZATION AND DEVELOPMENTAL EXPRESSIONDISTINGUISH THE ORPHAN NUCLEAR RECEPTORS NGFI-B, NURR1, AND NOR1" MOLECULAR ENDOCRINOLOGY, BALTIMORE, MD, US, 1996, pages 1656-1666, XP002919877 ISSN: 0888-8809 page 1657, right-hand column, line 55 -page 1659, left-hand column, line 40 ---	7,8
X	WO 96 21457 A (SALK INST FOR BIOLOGICAL STUDI) 18 July 1996 (1996-07-18) examples 2,7,8 claims 1,2,13-15 --- -/--	7,8

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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- *G* document member of the same patent family

Date of the actual completion of the international search

18 June 2002

Date of mailing of the international search report

02/07/2002

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Bayer, A

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 02/00744

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9621457	A	18-07-1996	AU 4530496 A	31-07-1996
			CA 2210248 A1	18-07-1996
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INTERNATIONAL SEARCH REPORT

International Application No

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PERLMANN T ET AL: "A NOVEL PATHWAY FOR VITAMIN A SIGNALING MEDIATED BY RXR HETERODIMERIZATION WITH NGFI-B AND NURR1" GENES AND DEVELOPMENT, COLD SPRING HARBOR, NY, US, vol. 9, no. 7, 1 April 1995 (1995-04-01), pages 769-782, XP002061503 ISSN: 0890-9369 the whole document especially page 773 right-hand column lines 9-29 and lines 46-52, page 778 left-hand column lines 7-11 -----	1-8